The Immune Response to Class I–Associated Tumor-Specific Cutaneous T-Cell Lymphoma Antigens

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In order to determine whether the neoplastic T cells from patients with cutaneous T-cell lymphoma express tumor-specific antigens that can serve as the targets of an immune response, we took advantage of family-specific monoclonal antibodies, magnetic bead technology, and recombinant cytokines, which provided the previously precluded ability to isolate and expand populations of purified tumor and autologous CD8 cytotoxic T cells. Four patients with advanced cutaneous T-cell lymphoma had CD8 cells that specifically killed autologous tumor in a class I limited fashion. Tumor cell cytolysis could be specifically enhanced by pre-culture with autologous γ-irradiated tumor. The cytolytic T cells produced tumor necrosis factor-α in response to stimulation with autologous tumor. The presence of tumor-specific cytotoxic T cells recognizing distinctive class I associated molecules on cutaneous T-cell lymphoma tumor cells suggests that infiltration of early lesions by CD8 cells reflects host immunity to the neoplasm. These studies provide the foundation for the development of tumor vaccines through the use of cytotoxic T cells to isolate and characterize tumor-associated cutaneous T-cell lymphoma peptides. ***Key words: cytotoxic T cells/immunotherapy/T-cell leukemia/tumor-associated peptides. J Invest Dermatol 107:392–397, 1996***

Cutaneous T-cell lymphoma (CTCL) is a relatively common clonal malignancy of "cutaneous T cells" that express a memory (CD45Ro) helper/inducer (CD4) phenotype and have a distinctive tendency to localize in the epidermis (Edelson *et al*, 1979; Edelson, 1980; Nickoloff and Griffiths, 1990; Terhune and Cooper, 1993). Like their normal counterparts, CTCL cells regularly display the "cutaneous lymphoid antigen," the ligand for endothelial-leukocyte cell adhesion molecule-1 expressed by dermal endothelial cells in inflamed skin (Picker *et al*, 1990). Expression of cutaneous lymphiod antigen CLA may contribute to the propensity of these malignant cells to home to the epidermis during the early stages of the disease (Picker *et al*, 1991).

As part of the natural course of CTCL, progressively more poorly differentiated and aggressive subclones of malignant cells become dominant, exhibiting diminished affinity for skin and greater metastatic potential (Edelson, 1980). Whereas in epidermotropic CTCL infiltrates nonmalignant mononuclear cells, including numerous $CD8^+$ T cells, predominate, more advanced cutaneous lesions are composed almost entirely of malignant cells (Wood *et al*, 1991; Bagot *et al*, 1992). This finding suggests that dissemination of CTCL results from the diminished expression of tumor antigens and/or a failure of the immune response to contain and destroy the malignant cells.

This premise is supported by findings in a large number of leukemic CTCL patients treated with extracorporeal photochemotherapy (Edelson *et al*, 1987; Heald *et al*, 1992), where response to treatment is apparently dependent on the presence of $CD8^+$ T cells, which increase in number in patients who are in induced remission. A complete response to therapy is more likely to occur in those patients in whom a residual CD8 population is present (Heald *et al*, 1992). Animal models of this therapy reveal that the immunoregulatory responses generated after reinfusion of phototreated activated lymphocytes are mediated by $CD8^+$ cytotoxic T lymphocytes (Perez *et al*, 1989). These observations have raised the possibility that CTCL cells bear tumor-specific antigens associated at the cell surface with class I major histocompatibility molecules.

Our novel observation that purified populations of CD8 cells selectively target autologous tumor cells, through a class I limited cytolytic response, was made possible through two recent advances: our ability to isolate large numbers of CTCL cells using highly selective monoclonal antibodies (Charley *et al*, 1990) and the availability of patients responding to immunotherapy for their CTCL (Edelson *et al*, 1987; Heald *et al*, 1992). The cytotoxic T-cell responses are patient specific and can be selectively enhanced by incubation with factors released from autologous tumor cells. The CD8 lines also produce tumor necrosis factor-α (TNF-α) in a tumor-specific fashion, and this cytokine may also play a role in the immune response to CTCL tumor cells.

MATERIALS AND METHODS

Isolation and Purification of Tumor and CD8 Cells To isolate large numbers of tumor and CD8 cells, leukocytes were enriched from the peripheral blood of CTCL patients, after informed consent (in accordance with institutional investigational review board policy), during therapeutic leukaphereses. The lymphocyte population isolated by ficoll hypaque flotation (Boyum, 1968) routinely yielded between 10^8 and 10^9 mononuclear leukocytes per pheresis. Excess cells were cryopreserved. Tumor cells were isolated with monoclonal antibodies reactive with the variable region of the β- or α-chain (V_β, V_α) of the T-cell receptor (T Cell Diagnostics, Woburn, MA). Approximately, 100×10^6 leukocytes/100 μl of RPMI 1640 (GIBCO, Gaithersburg, MD) were incubated with 200 μl of a 150 μg/ml solution of the appropriate anti-V region monoclonal antibody for 30 min at 4°C. The cells were washed and reincubated with magnetic sheep anti-mouse Ig beads (Dynal, Lake Success, NY; 1 ml of beads ~3

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beads/cell) for 1 h at 4°C on a tube rotator. Tumor cells that had bound magnetic beads were separated by placing the tube in a magnet and allowing the cells to adhere to the tube wall. Negative cells were aspirated and reserved for CD8 isolation.

The tumor cells were released from the magnetic field, washed, and incubated overnight at 37°C, in RPMI 1640 containing 20% fetal bovine serum (GIBCO), and 5 ng interleukin 7 per ml (IL-7, Genzyme, Cambridge, MA) to allow release of the magnetic beads through modulation of the surface receptor. The beads were removed by magnetic separation, and the tumor cells were cultured in media containing IL-7. Tumor cell recovery varied from 40–70% of the initial available tumor population. Viability was routinely greater than 95%, as determined by trypan blue exclusion. The tumor negative cells were incubated with anti-CD8–conjugated magnetic beads (Dynal, Great Neck, NY; 2 ml of CD8 beads/65 $\times$ 10^6 cells in RPMI 1640, ~4 beads/cell) in a similar fashion to that described for the tumor cells. The $CD8^+$ cells were incubated in IL-2 (500 units/ml, Genzyme) and IL-7 (5 ng/ml) in RPMI 1640/10% human AB serum overnight to allow removal of the beads. The recovered cells were freed from the remaining beads and recultured with IL-2, IL-7, and an equivalent number of γ-irradiated autologous tumor cells (1500 rads, Cesium Irradiator). The cells were fed with fresh media and irradiated autologous tumor on a weekly basis. Because the CTCL patients have a depleted CD8 population, the CD8 cell recovery varied depending on the initial CD8 count from 1 to 4 $\times$ 10^6, per 100 $\times$ 10^6 leukocytes processed. Viability of the recovered cells was greater than 95%.

Phenotypic Analysis To determine the purity of the isolated cell populations, monoclonal antibody phenotyping was performed as previously described (Kung *et al*, 1981). In brief, 1 $\times$ 10^6 lymphocytes were incubated with 100 μl of the relevant monoclonal antibody at the predetermined optimal dilution (generally 1:10 to 1:40 dilution of a 1 mg per ml solution of antibody) for 30 min at 4°C. The cells were washed twice and reincubated with a fluorescein-conjugated rabbit anti-mouse IgG reagent (1:40 dilution, Cappel, Durham, NC) for 30 min at 4°C. Fluorescence was analyzed by passage through a cytofluorograph (Coulter, Excel, Hialeah, FL).

Cytotoxicity Assay To determine the cytolytic response, purified CD8 cells were cultured for 2–3 wk and then added to 4 $\times$ 10^4, ^{51}chromium labeled tumor cells/well (1 μCi ^{51}chromium/10 $\times$ 10^6 cells, 1 h, 37°C). Triplicate wells were assayed for each culture. The plates were incubated for 6 h at 37°C and then centrifuged. The supernatants (100 μl/well) were harvested and counted in a gamma counter. Percent specific cytotoxicity was calculated by:

$$\frac{E - BG}{100\% \text{ lysis} - BG} \times 100$$

where E = experimental, BG = background, and 100% lysis is obtained by detergent lysis. Nonspecific release of ^{51}chromium from labeled target cells did not exceed 15% of the amount of isotope retained in the labeled targets.

Inhibition of cytotoxicity was tested by the addition of monoclonal antibody W6/32 (1:10 dilution of culture supernatant). W6/32 (a gift from Dr. Peter Cresswell, Yale University, New Haven, CT) recognizes a monomorphic epitope expressed on all class I molecules. The antibody or murine ascites of the same isotype was incubated with ^{51}chromium-labeled tumor cells, for 30 min at 4°C. The cells were washed and plated as described for the standard assay.

Limiting Dilution Isolation of CD8 Clones To determine whether clonal populations of $CD8^+$ effector cells could be obtained from the peripheral blood of CTCL patients, limiting dilution cultures were established by plating the CD8 cells at 0.3 cells/well in a microtiter plate, over a feeder layer of 1 $\times$ 10^4 γ-irradiated autologous tumor cells/well. The cells were cultured in standard media, and when growth became evident (approximately 3 wk) the cells were fed on a weekly basis. Approximately 30% of the wells showed growth and 8–16 clones were expanded from each patient. When 1 $\times$ 10^6 cells were obtained per well, the cells were moved into larger 2-cc wells and fed on a weekly basis. In some cases, 1 $\times$ 10^6 cells could be expanded to 6 $\times$ 10^6 cells after 3 wk of culture.

Co-cultivation of Tumor Cells with γ-Irradiated Autologous Tumor To determine if γ-irradiated tumor cells release substances that serve to sensitize viable autologous tumor cells to cytolysis, tumor cells were γ-irradiated and co-cultured at a 1:1 ratio with autologous tumor in standard media, for 24 h at 37°C. The co-cultures were labeled with ^{51}chromium for use as target cells in cytotoxicity assays.

Concanavalin A Stimulation of Normal Mononuclear Cells To determine the specificity of CD8 effectors for autologous tumor, normal mononuclear cells were obtained by reserving the cells that remained after tumor and CD8 cell depletion. The cells were cultured for 3 d at 2 $\times$ 10^6 cells per ml in RPMI 1640 containing 15% fetal bovine serum and 40 μg concanavalin A per ml. The cells were harvested, washed, and labeled with ^{51}chromium for use as targets in cytotoxicity assays.

Table I. Phenotype of Cell Populations Purified From Patients with CTCL

Patient	Cell Population	Monoclonal Antibody (% Positive Cells)[a] CD3	CD4	CD8	$V_{\beta,\alpha}$
AR	MNL	98	98	2	92 V_β8a
	Purified tumor	—[b]	—	—	98
	Purified CD8	—	0	90	—
	Cultured CD8 Line 4 mo	—	—	72	—
SS	MNL	95	92	2	83 V_β8a
	Purified tumor	—	98	—	81
	Purified CD8	—	—	62	—
	Cultured CD8 Line 4 mo	—	—	82	—
JS	MNL	83	62	21	47 V_β8b
	Purified tumor	—	—	—	—
	Purified CD8	—	—	84	—
WW	MNL	95	89	5	87 V_α2
	Purified tumor	—	95	—	94
	Purified CD8	—	—	87	—

[a] Data represent the percentage of fluorescent cells obtained by cytoflourograph evaluation.
[b] Dashes indicate that the phenotyping was not done.

[^{3}H]Thymidine Uptake To determine whether CD8 cells were stimulated to divide after exposure to γ-irradiated autologous tumor, proliferation assays were established. Purified CD8 cells (100 μl/well of 2 $\times$ 10^6 cells per ml) were incubated alone with RPMI 1640/10% human AB serum and IL-2 (100 μl/well), or added to 100-μl samples of 2 $\times$ 10^6 cells per ml γ-irradiated autologous tumor cells and cultured, for 6 d under 5% CO_2, in a 37°C incubator. Tumor cells were γ-irradiated and incubated alone (100-μl aliquot of a 2 $\times$ 10^6 cells per ml suspension) in standard media (100 μl/well). Proliferation was monitored by addition of 1 μCi [^{3}H]thymidine per well, for 6 h. The cells were harvested with a PhD-automated cell harvester (Cambridge Technology, Cambridge, MA). The incorporation of radiolabel was evaluated by counting in a beta liquid scintillation counter (Packard Instruments, Downers Grove, IL).

Class I Expression The level of class I expression was determined with Quantum Simply Cellular microbeads (Flow Cytometry Standards Corp., San Juan, Puerto Rico). The beads were labeled with anti-class I monoclonal antibody W6/32 through Fc receptor binding. The beads were then labeled with a secondary fluorescein-conjugated rabbit anti-mouse reagent. The beads were analyzed in the cytofluorograph and channel numbers were assigned an antibody binding capacity value through the use of the Quickcalc software. This program allows calculation of the number of antibody molecules bound to an unknown cell population through comparison of the unknown to the bead calibration sample.

TNF-α ELISA CD8 lines were added to γ-irradiated autologous tumor at a 5:1 effector-to-target ratio and cultured for 18 h at 37°C. The supernatants were centrifuged to remove cells and debris (900 $\times$ *g*) and tested for TNF-α concentration, according to the manufacturer's instructions (R&D Systems, Minneapolis, MN).

RESULTS

Phenotype of the Isolated Cell Populations Cell populations isolated from four CTCL patients were phenotyped with monoclonal antibodies to ensure that the tumor cells and the $CD8^+$ cells were freed of contaminating cell types and expressed the appropriate cell surface markers **(Table I)**. The initial peripheral blood phenotype of these patients demonstrates the marked expansion of clonal T cells, from a normal value of 5% of a V_β^+ or $V\alpha^+$ T-cell family to 47–92%, and depletion of the $CD8^+$ population in three patients, from a normal of 25% to 2–5% **(Table I)**. Despite a greater than 85% decrease in the percentage of CD8 cells, it was routinely possible to recover and expand sufficient purified CD8 cells to perform the cytotoxicity experiments. In two patients, CD8 cells

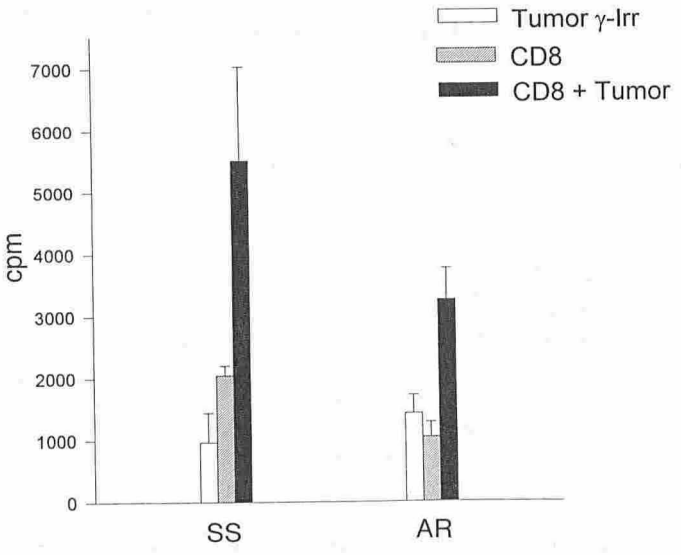

Figure 1. Tumor-driven proliferation of purified CD8 cells. Purified tumor cells (*open bars*) or CD8 cells (*hatched bars*) from patients SS and AR were cultured alone or co-cultivated (*solid bars*). Results represent the mean ± SD of ^{3}thymidine uptake in five replicate cultures.

were phenotyped after 4 mo in culture and the majority of the cells maintained CD8 expression.

CD8 Cell Expansion and Proliferation To demonstrate that CD8 cells were stimulated to expand and proliferate by incubation with cytokines and γ-irradiated autologous tumor, the number of CD8 cells was monitored over time. CD8 cells expanded in culture, from 2- to 5-fold over a 1–3 wk interval.

To demonstrate that the addition of γ-irradiated autologous tumor increased the rate of proliferation of isolated CD8 cells, uptake of ^{3}H-thymidine was evaluated. As shown in **Fig 1**, CD8 cells from patient SS demonstrated an increased proliferative response when γ-irradiated autologous tumor was added, in comparison to autologous tumor or CD8 cells incubated alone. CD8 cells isolated from patient AR also demonstrated a proliferative response to stimulation with autologous tumor, although at a reduced magnitude, in comparison to the response of CD8 cells from SS **(Fig 1)**.

Tumor Specific Cytolysis Purified and expanded polyclonal CD8 populations were evaluated for their capacity to lyse autologous purified tumor targets. Polyclonal CD8 lines isolated on three occasions, from patient AR reproducibly lysed autologous tumor cells **(Table II)**. In two experiments, two CD8 lines independently isolated from patient SS demonstrated 22% cytolysis of autologous tumor targets. Two polyclonal CD8 lines independently isolated from SS, 1 y later, demonstrated reduced cytolysis, at a higher effector to target ratio **(Table II)** suggesting diminished expression of tumor antigens. CD8 lines established from two other patients (JS and WW; **Tables II, III**) were also cytolytic for their autologous tumor. Specificity of the cytolytic response of CD8 lines from patients AR and SS is presented in **Fig 2** and in **Table II** for patients JS and WW.

In **Fig 2**, polyclonal CD8 cells from CTCL patient SS lysed autologous tumor targets but did not recognize tumor cells isolated from patient AR that shared the same T-cell receptor beta chain variable region (V_β8a). In a reciprocal experiment, CD8 cells from AR lysed his autologous tumor but did not lyse tumor cells isolated from patient SS **(Fig 2)**. In **Table II**, tumor cells from patient JS (V_β8b) were lysed by autologous polyclonal CD8 cells, whereas cells from AR were lysed at a low level. Mononuclear cells from patient AR, freed from tumor cells, were not recognized by polyclonal CD8 cells from JS. The results demonstrate that effector cells that recognize autologous tumor are specifically expanded in

Table II. Cytotoxicity of CD8 Lines and Clones Isolated from CTCL Patients

Patient	Exp[a,b]	E:T[a]	Target	Cytotoxicity
AR	n = 3	10:1	RT[c]	11.70 ± 2.15
SS	n = 2	5:1	ST[c]	22.00 ± 2.22
	n = 2	9:1	ST	13.50 ± 1.60
JS	n = 1	14:1	JST[c]	13.00 ± 1.14
			RT	4.00 ± 0.40
			RMNL[c]	0.00 ± 0.08
WW	n = 3	6:1	WT[c]	10.00 ± 1.60
			RB[c]	0.00 ± 0.0

[a] Abbreviations: Exp, number of experiments; E:T; effector:target ratio.
[b] Each experiment represents the mean ± SD of three replicate samples. Polyclonal CD8 lines were evaluated after independent isolation.
[c] RT, ST, JST, WT: Patients' autologous tumor cells; RMNL; patient AR's mononuclear leukocytes; RB, AR's EBV-transformed B cells.

the polyclonal CD8 population. In addition, an absence of alloreactivity for patient AR's EBV-transformed B cell line was demonstrated in three experiments with patient WW's CD8 lines **(Table II)**. Although the percentage of tumor cells lysed by the autologous CD8 lines is relatively small, the results were consistent in separate experiments performed with independently isolated CD8 lines from four different CTCL patients.

Because polyclonal CD8 cells are a heterogeneous population of effectors, cloned populations of CD8 cells were established so that the specificity of the response to autologous tumor could be studied further.

Tumor Specific Cytolysis Mediated by Cloned CD8 Effectors Cloned CD8 cells from 2 CTCL patients were tested for tumor specific cytolysis. Twenty-three clones were obtained from patient AR; 16 clones demonstrated sufficient growth for subsequent testing. When the clones were tested for tumor specific cytolysis, 6 clones demonstrated reactivity with autologous tumor cells. Limiting dilution cultures of patient SS yielded 22 clones, 5 of which were found to be cytotoxic for autologous tumor. The

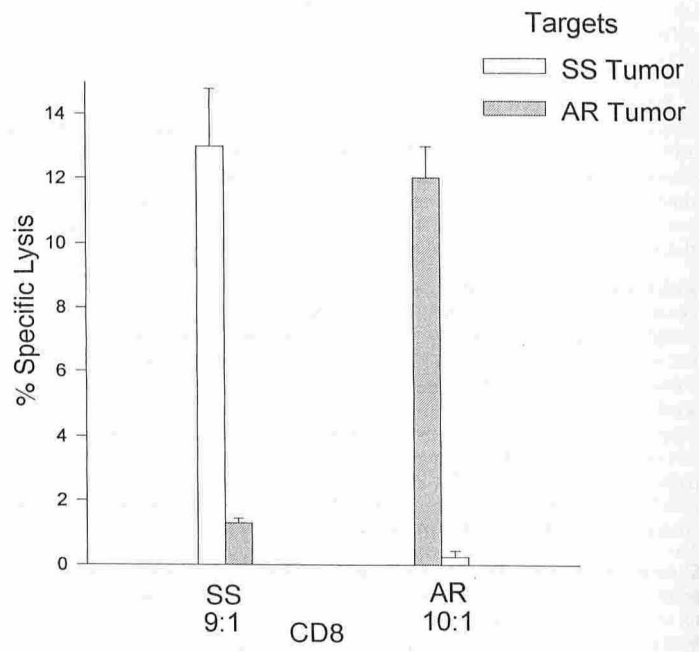

Figure 2. Tumor-specific cytotolysis of purified CD8 lines. Purified CD8 cells, from patients SS and AR, lyse autologous tumor targets (SS tumor, *open bars*; AR tumor, *hatched bars*) but do not lyse tumor cells purified from the other patient. Results represent the mean ± SD of three replicate samples.

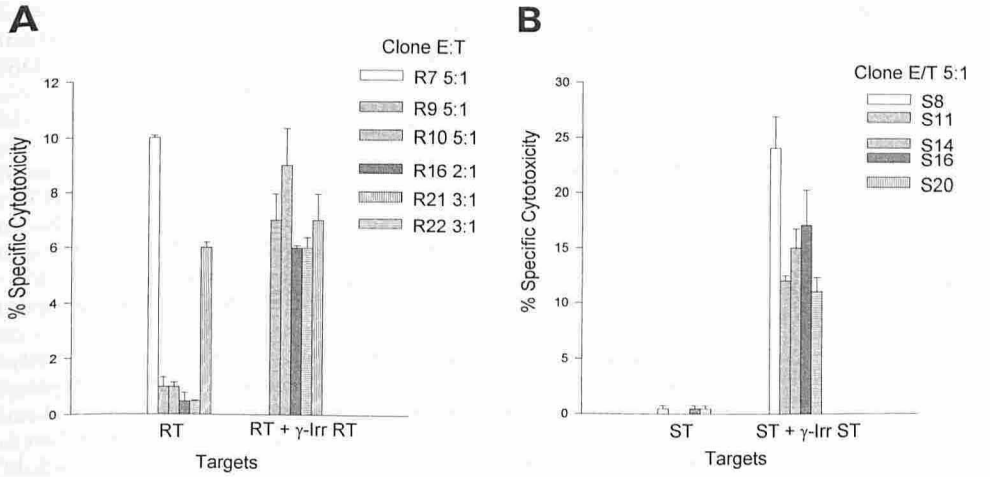

Figure 3. Tumor cytolysis by cloned CD8 cells frequently requires co-cultivation of tumor targets with γ-irradiated autologous tumor. Two of nine CD8 clones from patients AR (*a*) and SS (*b*) lyse unmodified tumor targets (RT, AR tumor; ST, SS tumor) whereas the remainder of the clones tested are cytolytic for autologous tumor co-cultivated with γ-irradiated tumor. The results represent the mean ± SD of three replicate samples.

cytolytic response of the clones derived from patients AR and SS are presented in **Fig 3*a*** and ***b***. Only 2 of 9 clones evaluated from both patients were able to lyse autologous tumor targets, however, overnight co-cultivation of the tumor cells with γ-irradiated autologous tumor resulted in restoration of clonal CD8 cytolysis.

Specificity of the enhanced cytolysis induced by co-cultivation with autologous γ-irradiated tumor but not allogeneic tumor is demonstrated in **Fig 4**. Unmodified autologous tumor cells were only minimally lysed. Whereas γ-irradiation of the tumor cells did not increase cytolysis, addition of γ-irradiated tumor cells and overnight co-cultivation resulted in improved lysis. Addition of γ-irradiated allogeneic tumor cells and overnight co-cultivation did not enhance cytolysis. In addition, CD8 clone S14 did not lyse activated, autologous normal lymphocytes (driven to become blasts by concanavalin A stimulation) indicating that activation of normal cells does not result in increased expression of the relevant target molecules.

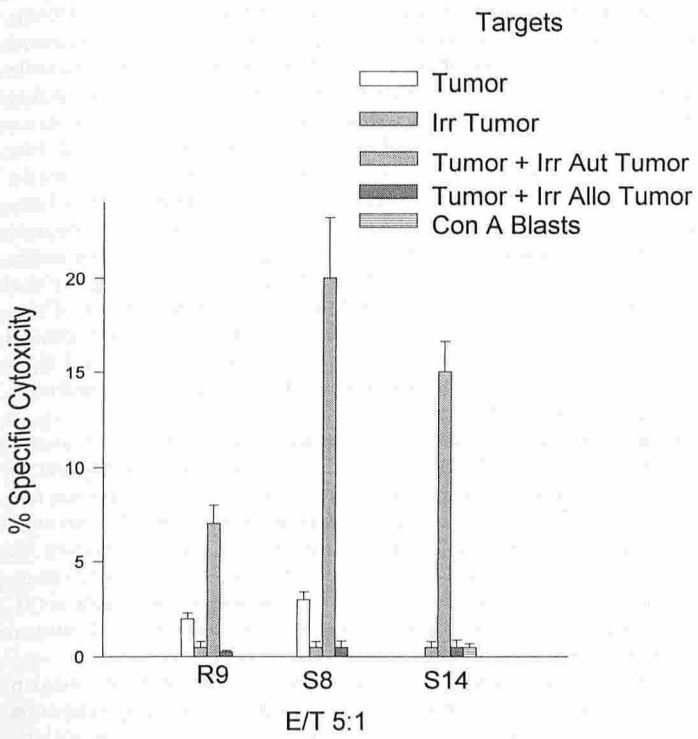

Figure 4. Specificity of the cloned CD8 cytolytic response for tumor co-cultivated with autologous γ-irradiated tumor. Autologous tumor is minimally lysed by CD8 clones (R9 and S8, *open bars*). The CD8 clones do not lyse γ-irradiated autologous tumor (*solid bars*). Co-cultivated of autologous tumor with γ-irradiated autologous tumor overnight (*hatched bars*), but not allogeneic tumor (*cross-hatched bars*) restores cytolysis. Clone S14 does not lyse autologous normal lymphocyte concanavalin A blasts (*striped bar*). The results represent the mean ± SD of three replicate samples.

Tumor Specific Cytolysis Requires Class I Expression on the Target Cells To determine whether the cytolysis was targeted to peptides held in class I molecules on the tumor cells, a monoclonal antibody reactive with class I major histocompatibility molecules, as a control irrelevant murine ascites, was added to block killing. The class I monoclonal antibody specifically inhibited tumor cytolysis mediated by CD8 lines derived from two patients **(Fig 5)**.

Class I Expression on Cultured CTCL Tumor Cells To determine whether reduced class I expression on the tumor cells impaired their ability to serve as targets, cultured CTCL cells were stained with the monoclonal anti-class I antibody, W6/32. Tumor cells from CTCL patients AR, SS, and WW expressed between 2

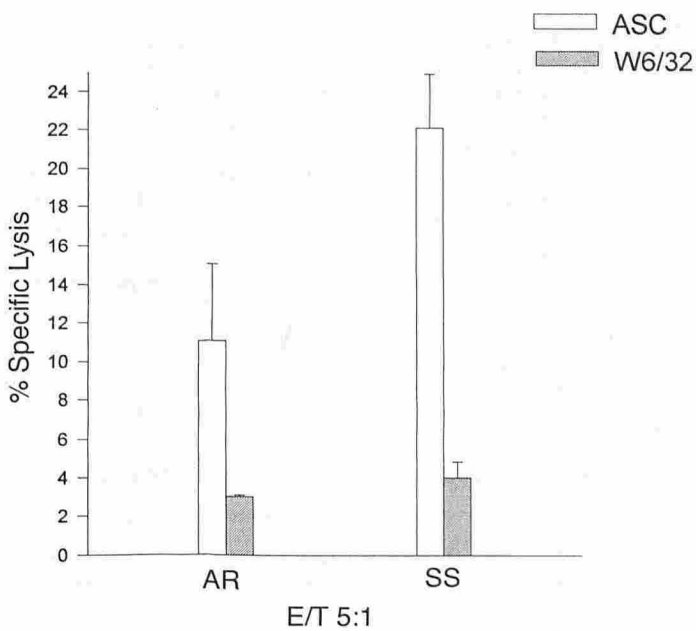

Figure 5. Blocking of cytolysis of polyclonal CD8 lines with class I specific monoclonal antibody. CD8 lines from patients AR and SS lyse autologous tumor in the presence of irrelevant mouse ascites (ASC, *open bar*). Addition of a monoclonal antibody to class I major histocompatibility complex molecules (*hatched bar*) blocks the cytolysis. The results represent the mean ± SD of three replicate samples.

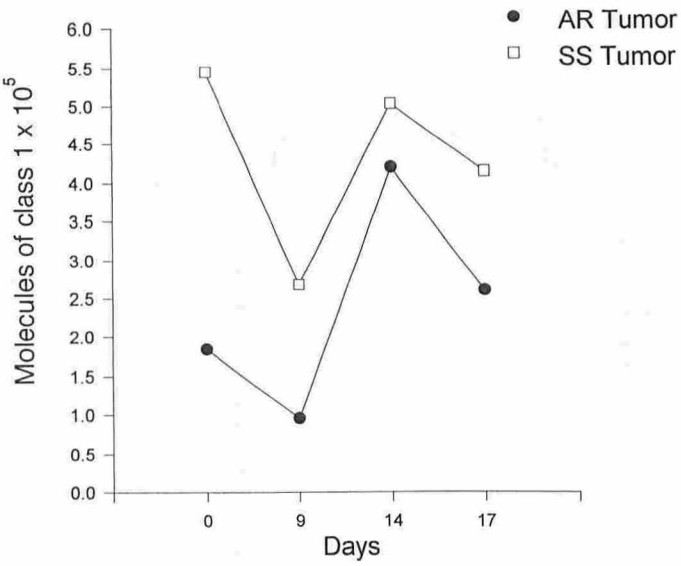

Figure 6. Variability of class I expression on cultured CTCL tumor cells. The number of molecules of class I major histocompatibility complex, detected by monoclonal antibody, varied on tumor cells from two CTCL patients over a 17-d culture period.

and 5.5×10^5 molecules of class I/cell. Normal expression of class I is approximately 10^5 molecules/cell (Bevan, 1984). In **Fig 6**, the expression of class I on the surface of tumor cells from patients AR and SS was compared over 2.5 wk of culture. Class I expression varied on the cell surface over time. At every time point tested, tumor cells from patient SS expressed more molecules of class I than tumor cells isolated from patient AR. In five replicate determinations, the average class I expression on SS tumor cells ($X = 3.74 \pm 1.79 \times 10^5$) was significantly increased ($p \leq 0.03$) above the class I expression detected on tumor cells from patient AR ($X = 2.37 \pm 1.19 \times 10^5$), supporting the contention that the increased cytolytic response to SS's tumor might relate to increased availability of class I-associated tumor peptides.

TNF-α Production by CD8 Lines in Response to CTCL Tumor To further characterize the response of CD8 lines to autologous tumor, culture supernatants from long-term lines were analyzed after 18 h of stimulation with autologous tumor. In **Table III**, the cytolytic response of three CD8 lines from patient WW was compared with the amount of TNF-α produced. Lines WW1 and 2 were cytolytic for autologous tumor and also secreted TNF-α into the culture supernatant. A non-cytolytic CD8 line WW3 produced no detectable TNF-α.

Long-term CD8 lines independently derived from patients AR and SS produced stable or increasing levels of TNF-α, in response to stimulation with autologous γ-irradiated tumor, over a 2 mo culture period. Co-cultivation of AR's or SS's CD8 lines with γ-irradiated tumor from the unrelated CTCL patient did not stimulate production of detectable levels of TNF-α. When γ-irradiated tumor or CD8 lines were cultivated alone, no TNF-α was detected. As observed for the cytolysis studies, CD8 lines derived from patient SS always produced higher levels of TNF-α than CD8 lines isolated from the other patients **(Table III)**.

Table III. Production of TNF-α by CD8 Lines

Patient	Exp[a]	E:T[a]	Cytotoxcity	TNF-α (pg/ml)
WW	1	3:1	23% ± 2.3	30
	2	7:1	18% ± 0.4	23
	3	4:1	0.5% ± 0.0	0
AR	1 mo	5:1	—[b]	29 ± 18.8[c]
	2 mo		—	47 ± 3.6
SS	1 mo	5:1	—	54 ± 11.9
	2 mo		—	77 ± 27.3

[a] Abbreviations: as in Table II.
[b] Dashes represent experiment not done.
[c] Mean ± SD of three determinations.

DISCUSSION

Tumor-specific immunity mediated by autologous cytotoxic T cells is a hallmark of tumor immunity (Young and Inaba, 1996). The frequency of tumor reactive cytotoxic T cells, however, has been demonstrated to be extremely low and variable (Massocchi *et al*, 1994). This deficit in cytotoxic T-cell precursors may result from reduced induction due to low expression of relevant tumor epitopes on the malignant cells.

Patients with adult T-cell leukemia, a malignancy of virally infected T cells, have cytotoxic T cells capable of lysing autologous tumor cells if sufficient epitopes of the viral protein are displayed on the cell surface (Kannagi *et al*, 1993). Although the levels of class I molecules were sufficient on the tumor cells, freshly isolated adult T-cell leukemia cells were only minimally lysed by autologous cytotoxic T cells *in vitro*. If the tumor cells were cultured with lectin or exogenous HTLV-1 tax peptide, the level of cytolysis increased substantially. Therefore, escape from immune surveillance in adult T-cell leukemia may be mediated by insufficient expression of target peptides on the tumor cell surface, despite the presence of a functional cytotoxic T-cell component and adequate expression of class I molecules.

Tumor-specific peptides on CTCL cells, presented in class I major histocompatibility complex molecules, are derived from cytoplasmic antigens processed for cell surface display by the endogenous pathway (Rotzschke and Falk, 1991). Endogenously synthesized peptides typically 8–10 amino acids in length, bind to the class I major histocompatibility complex molecules in the endoplasmic reticulum and are transported to the Golgi for export to the cell surface (Rotzschke and Falk, 1991; Kelly *et al*, 1992). Specific CD8 T cells recognize these peptide-class I complexes and target the cell for lysis (Massocchi *et al*, 1994). In this manner, cells expressing class I-associated peptides derived from viruses, point mutations, or clone specific T-cell receptors can be eliminated.

An immune response to the neoplastic lymphocytes in CTCL has not been previously demonstrated. The current studies were made possible by the use of family specific monoclonal antibodies (Charley *et al*, 1990), which can serve as an almost tumor-specific reagent permitting isolation of highly purified populations of tumor cells, which can be stimulated to proliferate for prolonged periods by the cytokine IL-7 (Dalloul *et al*, 1992). Expansion and cloning of the CD8 effector cells has been potentiated by the purified tumor stimulus and the observation that cytokines such as IL-2, and IL-7 promote cytotoxic T cell growth while preserving tumor specificity (Lynch and Miller, 1994).

Our results show that tumor-specific effector cells exist in the peripheral blood of CTCL patients despite the relative depletion of the CD8 compartment. These effectors recognize autologous tumor in a selective fashion and mediate tumor cell death, through cytolysis. Therefore, the limited ability of the immune system to control the spread of CTCL, in these subjects, does not appear to result from a deficit in the function of tumor-specific cytotoxic T lymphocytes but probably reflects reduced expression of tumor peptides on the malignant T cells.

The cytolysis observed in this system is unlikely to be due to natural killer cells because neither resting nor mitogen-activated normal lymphocytes were lysed while tumor targets were killed. Moreover, the cytolysis was blocked by class I-specific antibody and allogeneic tumor was not lysed (Falk *et al*, 1995). Therefore, the tumor cytolysis observed in these experiments was class I targeted, restricted to the tumor population, and not alloreactive.

The capacity of the tumor cells to be lysed depends on their ability to express class I histocompatibility molecules that present the relevant tumor epitope to the cytotoxic T cells (Kannagi *et al*, 1993; Massocchi *et al*, 1994). Our studies of class

I expression on the tumor cells reveal that the level varies in different patients and at different times on tumor cells cultured from the same patient. Tumor cells from patient SS, however, always expressed more molecules of class I than patient AR. Moreover, these cells were always lysed at a higher level than tumor cells isolated from AR and CD8 lines from SS produced higher amounts of TNF-α. These results suggest that tumor cells that express high levels of class I molecules have more of these molecules filled with the appropriate peptide that can be recognized by tumor-specific cytotoxic T lymphocytes.

Whereas the CD8 lines were able to lyse unmodified tumor cells, the cloned cells required the pre-culture of the tumor targets with autologous γ-irradiated tumor. This result may reflect recognition by the cloned cells of specific tumor peptides expressed in a limited fashion on the tumor cells. The polyclonal CD8 lines contain a variety of effectors so that multiple tumor peptides, some of which may be more prevalent on the tumor surface, are recognized. Therefore, polyclonal CD8 cells will be able to lyse tumor cells that express only low levels of some of the relevant peptides, because effectors specific for other more prevalent peptides are available.

Support for the observation of diminished cytolysis of tumor targets by cytotoxic T cells raised against these targets comes from the studies of CD8 cells expanded against Epstein Barr virus transformed B cell lines. The CD8 cells failed to lyse these targets unless the cells were superinfected with recombinant vaccinia virus expressing the appropriate Epstein Barr virus protein or incubated with the appropriate peptide epitope (Hill *et al*, 1995). These studies demonstrate that antigenic density may determine whether a target cell is susceptible to cytolysis.

Therefore, the factor that consistently limited the level of cytolysis mediated by CD8 cells derived from CTCL patients may be a deficit in the tumor population that results from reduced class I expression of relevant tumor antigen epitopes. Co-cultivation of γ-irradiated cells with autologous tumor may provide transfer of sensitizing peptides from the radiation damaged dying tumor cells to class I molecules on the viable tumor cells. Support for this hypothesis comes from the studies with adult T-cell leukemia tumor cells where exogenous peptide was able to sensitize tumor cells to cytolysis (Kannagi *et al*, 1993).

CD8 induced tumor cell death may be mediated by two mechanisms: necrosis or apoptosis (Zychlinsky *et al*, 1991). The production of TNF-α by tumor stimulated CD8 lines indicates that in addition to rapid cytolysis a more prolonged method of tumor cell death may also be operative. Binding of TNF-α to its receptor results in transduction of a death signal that may require 2–3 d to develop. The TNF-α studies confirm that CD8 effectors are triggered in a tumor-specific fashion.

Increased TNF-α production has also been described in macrophages isolated from CTCL patients undergoing photopheresis (Vowels *et al*, 1992). Macrophages are unlikely to contribute to the TNF-α production observed in our system because highly purified cell populations were tested and co-purified macrophages would have been depleted by plastic adherence during the long periods of *in vitro* cultivation (1–3 mo) of the isolated tumor and CD8 populations.

Our studies are an initial step towards isolation and characterization of the class I peptides important for priming the anti-tumor response in CTCL. Potential candidate peptides include retroviral antigens such as HTLV-I, products of normal genes that have undergone point mutations, or clone-specific T-cell receptor peptides. Unique peptides in an individual tumor cell may arise from frame shift mutations that give rise to new segments of a protein. Mutations resulting in new sequences of amino acids that produce novel peptides and can be presented by class I molecules have been shown to induce a vigorous cytotoxic T-cell response (Townsend *et al*, 1994).

Identification of peptide antigens will not only add to our knowledge of tumor immunobiology in CTCL, but also provide information that will help in the development of peptide vaccine therapies. Our studies demonstrate that it should be possible to positively affect the immune response in CTCL when the CD8 component is present, functional, and can be expanded with appropriate stimulation.

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